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Identification of a molecular weight 43,000 protein kinase in acetylcholine receptor-enriched membranes

(phosphorylation/ATP photoaffinity ligand)

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ABSTRACT **A photoaffinity ATP ligand is used to identify the protein kinase present in acetylcholine receptor-enriched membranes from *Torpedo californica*. Incubation of these membranes with 8-azido-[α-^{32}P]ATP and subsequent irradiation with UV light resulted in covalent labeling of a major band of M_r 43,000. Alkali-stripped membranes that show a selective reduction in the M_r 43,000 polypeptide also show a corresponding reduction in incorporation of photoaffinity label. In addition, the neutralized alkaline extract also showed one band at M_r 43,000 when labeled with the photoaffinity ligand. After alkali extraction, endogenous protein kinase activity decreased in the membranes in proportion to the loss of M_r 43,000 peptide. Moreover, the alkaline extract was able to phosphorylate casein in an exogenous assay system. These results suggest that a M_r 43,000 polypeptide in acetylcholine receptor-enriched membranes is the acetylcholine receptor kinase.**

The structure and function of the acetylcholine receptor (AcChoR) have been studied extensively in receptor-enriched membranes prepared from the electric organ of *Torpedo californica*. However, little is known about the function of other postsynaptic membrane components. Purified postsynaptic membranes from *T. californica* exhibit six major polypeptides on NaDodSO$_4$/polyacrylamide gel electrophoresis (1, 2). Four of these polypeptides are subunits of the AcChoR with molecular weights of 40,000, 50,000, 58,000, and 65,000 (α, β, γ and δ, respectively). The other two polypeptides are of M_r 43,000 and 90,000. The M_r 90,000 polypeptide is the Na/K ATPase present in these membranes and is similar to Na/K ATPase from other sources (3). However, the function of the M_r 43,000 is unknown. This polypeptide is a major component of receptor-enriched membranes even when prepared by different methods in different laboratories (see Fig. 3 and refs. 1, 2, and 4). The M_r 43,000 polypeptide can be selectively separated from the membrane by extraction at pH 11 for 30 min (1). Antibodies prepared against an alkaline extract show that the M_r 43,000 polypeptide is localized almost exclusively at the postsynaptic membrane in *Torpedo* electrocytes and the mammalian neuromuscular junction (5). These data suggest that the M_r 43,000 polypeptide is an important component of the nicotinic synapse.

We have shown that the β, γ, and δ subunits of the AcChoR are phosphorylated *in situ* by an endogenous protein kinase when AcChoR-enriched membranes are incubated with [γ-^{32}P]ATP and Mg^{2+} (6). The membranes also show protein phosphatase activity that dephosphorylates the membrane-bound AcChoR (7). These results indicate that both protein kinase and protein phosphatase activities are intimately associated with the AcChoR in receptor-enriched membranes. However, these enzymes have not yet been identified. We now present evidence that an alkali-extractable M_r 43,000 polypeptide associated with the AcChoR binds ATP and appears to have protein kinase activity.

MATERIALS AND METHODS

Purification of Postsynaptic Membranes. Receptor-enriched membranes from *T. californica* were prepared according to the method of Elliott *et al.* (8). One hundred fifty grams of frozen electric organ was homogenized in 150 ml of buffer (0.4 M NaCl/10 mM NaPO$_4$/10 mM EDTA/10 mM EGTA/0.02% NaN$_3$/10 mM phenylmethylsulfonyl fluoride) for 2 min at top speed in a Waring Blendor. Aliquots were rehomogenized for four 30-sec periods at top speed in a Virtis type 23 homogenizer. The homogenate was centrifuged at 4,000 × *g* for 10 min and the supernatant was passed through two layers of cheesecloth. The effluent was spun for 1 hr at 100,000 × *g*. The resulting pellet was suspended in 10 mM NaPO$_4$/1.0 mM EDTA/0.02% NaN$_3$ (PE buffer) and homogenized for two 30-sec periods in the Virtis. Sucrose and NaCl were then added to final concentrations of 30% sucrose (wt/wt) and 0.4 M NaCl. Aliquots (15 ml) were layered over a gradient consisting of 5 ml of 50% sucrose (wt/wt) and 12 ml of 35% sucrose (wt/wt) in 0.4 M NaCl/10 mM NaPO$_4$/1 mM EDTA/0.02% NaN$_3$. The samples were overlaid with PE buffer containing 0.4 M NaCl and centrifuged in a Beckman vertical Ti 50 rotor at 45,000 rpm for 60 min with slow acceleration and deceleration. The middle band contained AcChoR-enriched membrane vesicles. These fractions were pooled, diluted with 2 vol of 0.4 M NaCl/10 mM Tris·HCl/1 mM EDTA, pH 7.4 (NTE buffer), and centrifuged at 100,000 × *g* for 60 min. Pellets were suspended in NTE buffer and stored in liquid nitrogen until used. The membranes contained 3–5 nmol of α-bungarotoxin binding sites per mg of protein.

Alkali Extraction of Membranes. AcChoR-enriched membranes were alkali stripped by two different procedures. To study the membranes after alkali treatment, 7–10 mg of AcChoR-enriched membranes was diluted with 25 ml of H$_2$O and the pH was adjusted to 11 with 1 M NaOH. Membranes were incubated at 4°C for 60 min and then centrifuged at 100,000 × *g* for 60 min and suspended in NTE buffer.

To obtain enough alkali-extractable protein for study, AcChoR-enriched membranes were extracted at pH 11 for 30 min at 4°C at a protein concentration of 10 mg/ml and then centrifuged for 20 min in a Beckman Airfuge. The extract was adjusted to pH 7.4 and the standard NTE buffer concentration.

Photoaffinity Labeling of Membranes and the Alkaline Extract. Various amounts of AcChoR-enriched membranes, neutralized alkaline extract, or alkali-stripped membranes were incubated in a final vol of 100 μl with 1 μCi of 8-azido-[α-^{32}P]-ATP (1 Ci = 37 GBq) in protein kinase buffer (1.6 mM ouabain/

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Abbreviation: AcChoR, acetylcholine receptor.

10 mM $MnCl_2$/100 mM NaF/300 mM NaCl/10 mM Tris·HCl, pH 7.4) in the presence or absence of 0.02% Triton X-100.

Samples were incubated in microtiter plates for 5 min at room temperature and then placed on ice and irradiated for 1 min at a distance of 8 cm from the UV source [General Electric germicidal lamp (GE shortwave)]. The reaction was stopped by the addition of $NaDodSO_4$/EDTA/2-mercaptoethanol as described (9) and the proteins were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis (10). Gels were stained with Coomassie blue R and autoradiographed for 4–24 hr with two Cronex intensifying screens.

Protein Kinase Activity. Endogenous protein kinase activity was determined as described (9). AcChoR-enriched membranes or alkali-extracted membranes were incubated in protein kinase buffer containing 0.02% Triton X-100 for 5 min at room temperature with 5 μM [γ-^{32}P]ATP (2 μCi). The reaction was stopped as described above and the samples were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The dried gels were then autoradiographed overnight and developed with Cronex screens.

Exogenous protein kinase activity was assayed by using casein as phosphate acceptor as described (11). The alkaline extract was adjusted to a final concentration of 15 mM Tris·HCl (pH 7.4). Phosphorylation of casein was carried out in a final vol of 100 μl of 1.6 mM ouabain/10 mM $MnCl_2$/100 mM NaF/15 mM Tris·HCl, pH 7.4/50 μM [γ-^{32}P]ATP (2 μCi) containing 1.8 mg of casein and various amounts of alkaline extract. Samples were incubated for 30 min at 37°C. The reaction was stopped by the addition of perchloric acid as described (11), the precipitates were collected on Whatman GF/C filters, and radioactivity was determined in 10 ml of Filtron X scintillation fluid (National Diagnostics, Somerville, NJ).

Materials. 8-Azido-[α-^{32}P] ATP (specific activity, 97 Ci/mmol) was purchased as the tetratriethylammonium salt from Schwarz/Mann. [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Amersham. Casein was purchased from Miles and treated as described (11) before use.

RESULTS

8-Azido-ATP (12), a photoaffinity analog of ATP, has been used to identify protein kinases. To determine whether this analog would be useful in identifying the receptor kinase, we searched for evidence that 8-azido-ATP inhibits AcChoR phosphorylation. AcChoR-enriched membranes were incubated for 20 min with 8-azido-ATP at various concentrations and then assayed for protein kinase activity with [γ-^{32}P]ATP. Fig. 1 shows that, at higher concentrations of 8-azido-ATP, there were corresponding decreases in kinase activity. These results suggest that 8-azido-ATP reacts with an ATP binding site to inhibit the receptor kinase.

To determine which polypeptide in AcChoR-enriched membranes is the receptor kinase, we used 8-azido-[α-^{32}P]ATP. Because the ^{32}P label is in the α position, this allows us to use autoradiography to identify a covalently labeled peptide that has an ATP binding site. As shown in Fig. 2, a major polypeptide of M_r 43,000 is labeled in AcChoR-enriched membranes after irradiation with UV light for 1, 2, or 5 min. No labeling was observed in the absence of UV light or in the presence of excess unlabeled ATP. On longer exposure of the film, a labeled polypeptide of M_r 90,000 that corresponds to the active subunit of the Na/K ATPase is seen. However, 20 times more label was incorporated into the M_r 43,000 polypeptide than into the ATPase. The two minor labeled peptides of low molecular weight appear to be degradation products of the M_r 43,000 band since they are absent when membranes are used shortly after preparation.

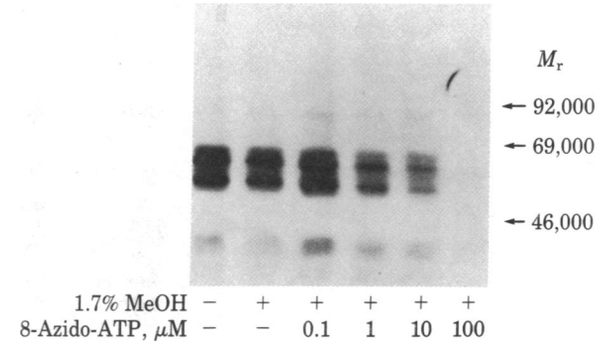

FIG. 1. Endogenous protein kinase activity. AcChoR-enriched membranes were incubated as indicated with 8-azido-ATP for 20 min at room temperature. Then, 5 μM [γ-^{32}P]ATP (2 μCi) was added and the samples were incubated further for 5 min. The reaction was stopped, and the samples were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The dried gels were autoradiographed overnight and developed. Since the 8-azido-ATP was diluted from 100% methanol, a control is shown for the effects of 1.7% methanol (final methanol concentration in all cases) on protein kinase activity.

The labeled polypeptide corresponds in molecular weight to the major M_r 43,000 polypeptide that is extractable from receptor-enriched membranes by treatment at pH 11 (1). Therefore, we determined whether alkali extraction affected the labeling of membranes. Coomassie blue staining of a $NaDodSO_4$/polyacrylamide electrophoresis gel (Fig. 3*A*) shows that the major protein extracted from the membranes is the M_r 43,000 polypeptide (arrow). An autoradiogram of this gel (Fig. 3*B*) shows that there is a concomitant reduction of photoaffinity labeling of the M_r 43,000 polypeptide (arrow), suggesting that the alkali-extractable M_r 43,000 polypeptide is being labeled by the photoaffinity ligand.

To verify that the alkali-extractable M_r 43,000 polypeptide has an ATP binding site, we incubated the neutralized alkaline extract with 8-azido-[α-^{32}P]ATP in the presence of UV light. Coomassie blue staining of a $NaDodSO_4$/polyacrylamide electrophoresis gel (Fig. 4*A*) shows that the major protein in the alkaline extract is the M_r 43,000 polypeptide. The autoradiogram of this gel (Fig. 4*B*) shows that, after neutralization, the M_r 43,000 polypeptide in the alkaline extract reacts with the ATP photoaffinity label (Fig. 4*B*, lanes k–n). This experiment also shows that low concentrations of Triton X-100 (0.02%) are required for labeling to occur in the membrane (lanes h–j). This

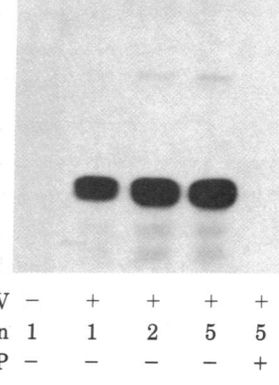

FIG. 2. Autoradiogram of a $NaDodSO_4$/polyacrylamide electrophoresis gel of AcChoR-enriched membranes that had been incubated with 1 μCi of 8-azido-[α-^{32}P]ATP (97 Ci/mmol) in NTE buffer containing 0.02% Triton X-100 for 5 min and then irradiated for the indicated times. Where indicated, the membranes were first incubated for 1 min with unlabeled 10 mM ATP. The major band is at M_r 43,000.

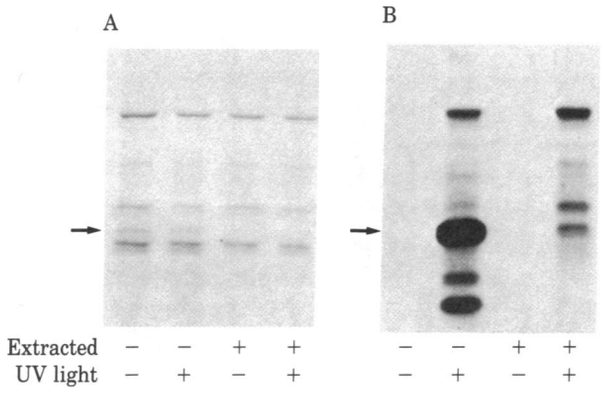

FIG. 3. (*A*) Coomassie blue-stained NaDodSO$_4$/polyacrylamide electrophoresis gel of either AcChoR-enriched membranes or alkali-extracted membranes (20 μg of protein) after incubation with 1 μCi of 8-azido-[α-^{32}P]ATP in NTE buffer containing 0.02% Triton for 5 min followed by incubation for 1 min in the presence or absence of UV light. (*B*) Autoradiogram of the gel shown in *A*. Arrows indicate the M_r 43,000 polypeptide.

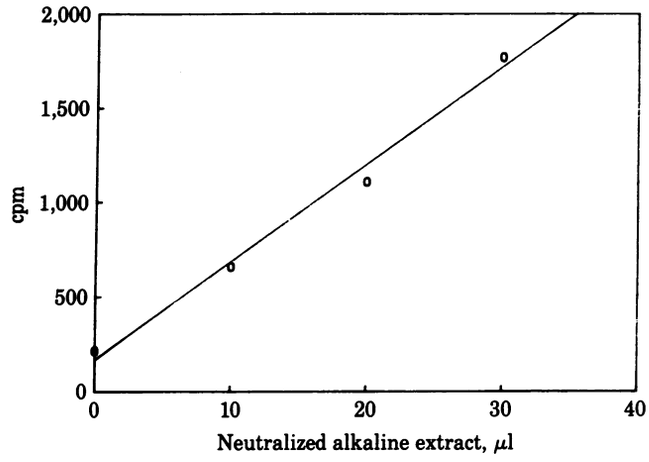

FIG. 5. Exogenous protein kinase activity. Incorporation of $^{32}PO_4$ into casein as a function of the amount of alkaline extract in the incubation medium. The amount of $^{32}PO_4$ incorporated was corrected by subtracting the endogenous phosphorylation of the alkaline extract and the casein blank from the experimental values.

is probably because most of the membranes are sealed vesicles and detergent permits ATP to reach the cytoplasmic face of the membrane (9). Triton is not necessary for photoaffinity labeling of the alkaline extract (Fig. 4*B*, lanes k and l).

Since the M_r 43,000 polypeptide binds ATP, it appeared likely that this polypeptide might also be a protein kinase. After alkali extraction, endogenous protein kinase activity decreased in the membranes in proportion to the loss of M_r 43,000 peptide (data not shown). This suggested that protein kinase activity was being extracted from the membrane. However, an alternative explanation is that alkali treatment inactivated the membrane kinase. Therefore, we needed to determine directly whether protein kinase activity was present in the alkaline extract. We have already shown that the protein kinase in the AcChoR-enriched membranes can phosphorylate casein in an exogenous phosphorylation assay (11). Therefore, we used this assay to search for protein kinase activity in the alkaline extract. Increasing amounts of the alkaline extract were added to casein in the presence of [γ-^{32}P]ATP. Fig. 5 shows that this produced a linear increase in casein phosphorylation. These results confirmed that the alkaline extract enriched in the M_r 43,000 polypeptide also contains protein kinase activity. The alkali-extractable kinase is inhibited by low concentrations of nonionic detergent (data not shown). Therefore, we could not determine whether the membrane-bound or the solubilized AcChoR is a substrate for the extracted kinase because both assays require the presence of detergent.

DISCUSSION

We have shown that AcChoR-enriched membranes contain a protein kinase activity that phosphorylates at least three subunits of the membrane-bound AcChoR (6). To identify this receptor kinase, we used a radioactively labeled photoaffinity derivative of ATP to react with the ATP recognition site of the enzyme. Incubation of AcChoR-enriched membranes with 8-azido-[α-^{32}P]ATP and subsequent irradiation with UV light resulted in the labeling of a major band of M_r 43,000. Alkali-stripped membranes that exhibit a selective reduction in the M_r 43,000 polypeptide also show a corresponding reduction in incorporation of photoaffinity label (Fig. 3). Moreover, the neutralized alkaline extract of these membranes also showed a band at M_r 43,000 when labeled with 8-azido-ATP (Fig. 4). These results indicate that an alkali-extractable M_r 43,000 polypeptide has a binding site for ATP.

To determine whether this ATP binding activity represents

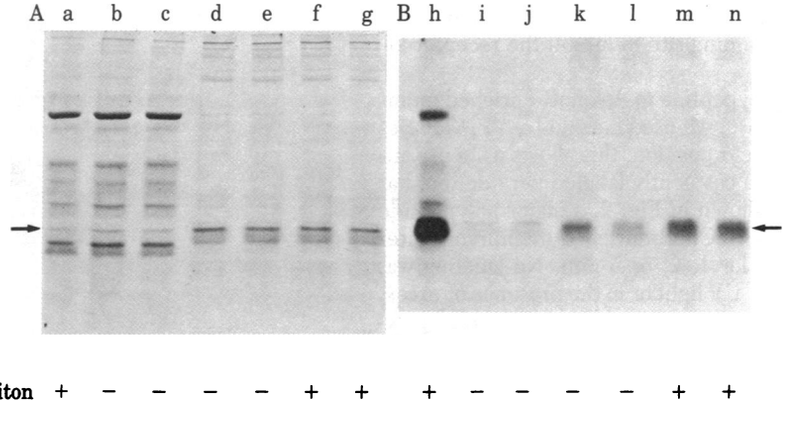

FIG. 4. (*A*) Coomassie blue-stained NaDodSO$_4$/polyacrylamide electrophoresis gel of either AcChoR-enriched membranes (lanes a–c) or the alkaline extract (lanes d–g) after incubation with 1 μCi of 8-azido-[α-^{32}P]ATP in NTE buffer in the presence or absence of 0.02% Triton for 5 min followed by irradiation for 1 min with UV light. Lanes b and c, d and e, and f and g are duplicate samples. (*B*) Autoradiogram of the gel shown in *A*. Arrows indicate the M_r 43,000 polypeptide.

the ATP binding site of a protein kinase, we measured protein kinase activity in the alkaline extract. We found that the alkali-extractable protein was able to phosphorylate casein in an exogenous protein kinase assay (Fig. 5). Since the M_r 43,000 peptide is virtually the only ATP binding protein in the alkaline extract, it is likely that a M_r 43,000 polypeptide in AcChoR-enriched membranes is the protein kinase.

It has been shown in our laboratory that the protein kinase that phosphorylates the membrane-bound AcChoR is located on the cytoplasmic face of the postsynaptic membrane. Wennogle and Changeux (4) and St. John *et al.* (13) have shown that a M_r 43,000 polypeptide that is the major component of the alkaline extract is also located on the cytoplasmic face of the membrane. The M_r 43,000 polypeptide that we label with 8-azido-[α-^{32}P]ATP appears to be located on the cytoplasmic face of the membrane since, in sealed vesicles, this reaction occurs only in the presence of detergent (Fig. 3). Taken together, these data support our hypothesis that the M_r 43,000 polypeptide in the postsynaptic membrane binds ATP and has protein kinase activity.

The M_r 43,000 polypeptide copurifies with AcChoR-enriched postsynaptic membranes and is a major component of these membranes (1, 2). This polypeptide can be removed from *Torpedo* membranes by extraction at pH 11 without altering either the binding of cholinergic ligands or cholinergic agonist-stimulated ion flux (1, 2). However, extraction of this polypeptide has effects on the structural properties of the AcChoR (1, 4, 14, 15). These results suggest that the M_r 43,000 polypeptide interacts in some way with the AcChoR. Froehner *et al.* (5) have prepared antibodies to the alkaline extract of *Torpedo* membranes and have shown that these antibodies react almost exclusively with the innervated surface of *Torpedo* electrocytes (5). Moreover, this antiserum crossreacts with a component of the rat neuromuscular junction that is highly concentrated at the synapse (5), suggesting that the M_r 43,000 peptide is also an important component of the mammalian neuromuscular junction.

The major finding in this study is that an alkali-extractable M_r 43,000 polypeptide binds ATP and has protein kinase activity. It is probable that the M_r 43,000 polypeptide that binds ATP is the receptor kinase that phosphorylates the AcChoR in the postsynaptic membrane. However, it remains to be determined whether the M_r 43,000 polypeptide labeled by the covalent ATP analog is the same major polypeptide observed in the alkaline extract. Nevertheless, identification of this receptor kinase will make it possible to learn more about regulation of the AcChoR at the nicotinic synapse.

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